Characterization of Primary T Helper Cell Activation and T Helper Cell Lines Stimulated by Hapten-modified, Cultured Langerhans Cells

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It has recently been shown that hapten-modified cultured Langerhans cells are able to activate small resting syngeneic L3T4$^+$ T helper cells from nonsensitized animals. Repeated stimulation of these T cells with hapten-modified cultured Langerhans cells leads to the establishment of L3T4$^+$ hapten-specific interleukin-4-producing T-cell lines. Here we report on further characteristics of primary hapten-dependent activation of L3T4$^+$ T cells and of T-cell lines derived from them. Dendritic cell-enriched spleen cells were as able as Langerhans cells to activate nonsensitized T helper cells after hapten modification. However, M12c, a major histocompatibility complex class II-positive B-cell line that was able to activate small, resting, allogeneic L3T4$^+$ T cells was not able to stimulate syngeneic T helper cells after hapten modification. Thy1$^+$ dendritic epidermal cells did not significantly affect the magnitude of primary T helper cell proliferation induced by cultured Langerhans cells. Restimulation of in vitro primed T helper cells with hapten-modified cultured Langerhans cells revealed the presence, within the primed T helper cell population, of activated cells with specificity to an unrelated hapten, suggesting that, in hapten-dependent T helper cell activation, hapten-nonspecific cells are activated along with those that are hapten specific. Restimulation of a hapten-specific long-term T helper cell subline using different antigen-presenting cell types demonstrates that factors other than major histocompatibility complex class II density or tissue derivation of the antigen-presenting cell play a role in the activation of T cells in vitro. Finally, we demonstrate that in vitro generated hapten-specific T helper cell lines may not show strict major histocompatibility complex restriction.
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We previously reported that cultured epidermal Langerhans' cells (cLC), after hapten modification, are capable of stimulating the proliferation of small, resting, L3T4$^+$ T helper cells (Th) from nonsensitized animals [1]. Primary hapten-induced Th proliferation was dependent on the presence of specific Th within the unprimed Th population; in addition, upon restimulation with hapten-modified spleen cells, the in vitro primed Th population responded in a hapten-specific manner. Primary hapten-dependent Th proliferation was blocked by antibodies to major histocompatibility complex (MHC) class II antigens, L3T4 and LFA-1. In a subsequent series of experiments, we demonstrated that L3T4$^+$ Th lines generated by repeated stimulation with hapten-modified cLC produce interleukin 4 but no detectable interleukin 2 (IL-2) [2]. Furthermore, these Th lines were able to stimulate IgE production in small, resting, nonprimed B cells after cognate interaction [2].

In the present report, we compare cLC to other antigen-presenting cells (APC) in the primary hapten-dependent proliferation assay, as well as in restimulation assays using previously activated Th. Furthermore, we describe the effect of Thy1$^+$ dendritic epidermal cells on primary Th proliferation. We demonstrate the existence of "nonspecific" Th activation that accompanies primary specific hapten-dependent Th activation. We also show that hapten-specific Th lines generated in vitro with cLC lack classical MHC restriction. Finally, we present a Th subline that responds to cLC and dendritic cell-enriched spleen cells but not to spleen cells or M12c cells.

MATERIALS AND METHODS

Preparation of Th Depleted of Autoreactivity As previously described [1], nylon wool nonadherent lymph node cells treated with anti-MHC class II (M5/114.15.2); American Type Culture Collection [ATCC]. Rockville, MD), anti-Lyt-2 (53-6.72; ATCC), mouse anti-rat kappa chain (MAR 18.5; ATCC) antibodies, and C′ (Cedarlane, Hornby, Ontario) were cultured at 6 x 10^6 cells/well with either 0.3 x 10^6 syngeneic cLC prepared as described [1] or 0.5 × 10^6 mitomycin-treated syngeneic M12c, a B-cell line derived from the Ia-bearing M12 B cell lymphoma [3] (kindly provided by Dr. Ronald Germain, Laboratory of Immunology, NIAID, NIH,

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Abbreviations:
APC: antigen-presenting cell
cLC: cultured epidermal langerhans' cells
IL-2: interleukin-2
FITC: fluorescein isothiocyanate
MHC: major histocompatibility complex
Th: T helper cells
TNP: trinitrophenyl

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Bethesda, MD) in 2 ml culture medium. Culture medium consisted of RPMI 1640 (Biofluids, Rockville, MD or Gibco, Grand Island, NY) containing 10% fetal calf serum (Biofluids), 100 U/ml penicillin (Gibco), 100 μg streptomycin (Gibco), 2 μg/ml fungizone (Gibco); 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), 50 μg/ml gentamycin (Gibco), 2 mM glutamine (Gibco), and 1 μg/ml indomethacin (Sigma). After 4 d of culture, activated Th cells were eliminated using bromodeoxiuridine, bisbenzimide, light irradiation, antiIL-2 receptor antibody, and complement, as described [1]. The remaining nonactivated Th were used for primary Th activation experiments.

Preparation of In Vivo Primed Th Animals were painted with 100 μl 7% trinitrochlorobenzene (4:1, acetone:olive oil) on the abdominal skin. Five to seven days later, the animals were killed. Lymphocytes from inguinal, axillary, and subscapular lymph nodes were prepared. After nylon-wool purification, the cells were treated with anti-MHC class II (M5/114.15.2) and anti-Lyt-2 (53-6.72) mouse anti-rat kappa chain (MAR 18.5) monoclonal antibodies and C′. The resulting cells were referred to as in vivo primed Th.

Preparation of Dendritic Cell-enriched Spleen Cells Spleen cells were incubated on plastic petri dishes (3 spleen/10-cm diameter plate; Costar, Cambridge, MA) in 10 ml culture medium. After 90 min, nonadherent cells were removed, and the plate was rinsed two times with fresh culture medium. The plates containing fresh culture medium were then incubated overnight at 37°C and 5% CO_2. The following day nonadherent cells were removed and spun over a 50% Percoll gradient (Pharmacia, Uppsala, Sweden) at 2000 *g* for 15 min at 4°C. The interface cells were recovered and washed three times.

Proliferation Assays Spleen cells, dendritic cell-enriched spleen cells, mitomycin-treated M12c, and cLC were either trinitrophenylated (TNP) or fluoreceinated by incubation with trinitrobenzene sulfonic acid (Eastman, Rochester, NY) or with fluorescein isothiocyanate (FITC) isomer I (Sigma) as described [1]. Spleen cells were irradiated at 3300 rad, and cLC and dendritic cell-enriched spleen cells at 1500 rad. For primary proliferation assays, the various stimulator cell populations were cultured with 10^5 Th depleted of autoreactivity or 10^5 freshly prepared allogeneic Th in 96-well flat-bottomed tissue culture plates (Costar) for 5 d. After [^{3}H]methylthymidine (1μCi/well; Amersham, Arlington Heights, IL) pulsing for the final 16 h, the cultures were harvested and assessed for incorporation of radioactivity into cells. Results are given as means of triplicate cultures ± 1 SEM.

Preparation of cLC and Thy1$^+$ Dendritic Epidermal T Cells cLC were prepared as described [1]. Epidermal cells prepared from trunk skin of C3H mice by trypsinization for 40 min at 37°C [4] were spun over a Ficoll gradient (3 parts 9% Ficoll 400 [Pharmacia], 2 parts Hypaque 90% [Winthrop, New York, NY]). The interface cells were washed and incubated with anti-class I (36.7.5) and anti-class II (10.2.16; both kindly provided by Dr. D. Sachs, Immunology Branch, NCI, NIH) monoclonal antibodies and then treated with low tox M rabbit complement (Cedarlane) 1:10 for 90 min. Thereafter, the cells were washed and spun over a Ficoll gradient (as above). The interface cells were washed and used for proliferation assays. The cells were 30%-60% Thy1$^+$ as assessed by indirect immunofluorescence with an FITC-conjugated anti-Thy1 antibody (Becton Dickinson, Mountain View, CA).

Flow Cytometric Analysis of Class II Intensity M12c, cLC, and dendritic cell-enriched spleen cells were incubated with FITC-conjugated MKD6 (anti-I-A^d) or 10.2.16 (anti-I-A^k) or with unconjugated antibodies and an FITC-conjugated goat F(ab)$_2$ anti-mouse IgG (Tago, Burlingame, CA). The washed cells were then analyzed in a flow cytometer (Becton Dickinson or Coulter, Hialeah, FL). Dead cells were excluded by propidium iodide staining. Ten thousand cells were scored for each sample. Results are given as number of events on the y axis and relative fluorescence intensity or fluorescence channel on the x axis.

RESULTS

Comparison of cLC, M12, and Spleen Cells Enriched for Dendritic Cells in Primary Lymphocyte Proliferation and Lymphocyte Restimulation Assays Th depleted of autoreactivity as described [1] using M12c cells were tested in a primary hapten-dependent proliferation assay using either cLC or M12c cells (Fig 1). Cultures using TNP-modified cLC stimulated vigorous primary Th proliferation (Fig 1*D*), whereas unmodified cLC led to minimal Th proliferation. No primary Th proliferation was seen using TNP-modified or unmodified M12 cells (Fig 1*A*). Both cLC and M12c stimulated freshly prepared allogeneic Th cells (Fig 1*B, E*) and were able to restimulate an in vitro generated TNP-specific Th line (Fig 1*C, F*).

TNP-modified dendritic-cell enriched spleen cells were fully capable of generating primary in vitro sensitization of Th depleted of autoreactivity (Fig 2). In addition, these cells could induce an allo-

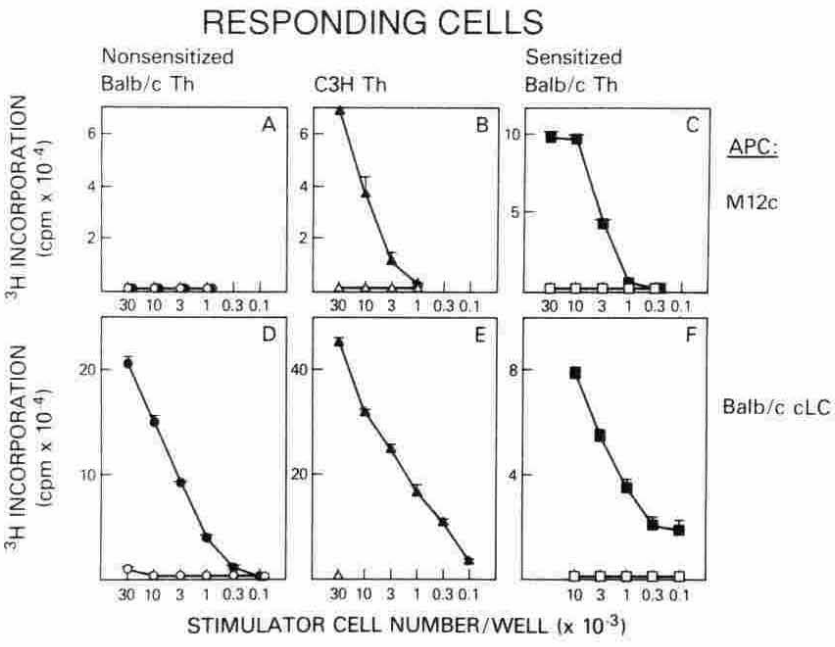

Figure 1. cLC but not M12c cells are able to stimulate syngeneic nonsensitized Th when hapten modified. Nonsensitized Balb/c Th (10^5) depleted of autoreactivity *(A, D, circles)*, 10^5 freshly prepared C3H Th *(B, E, solid triangles)*, or 10^4 in vitro sensitized Balb/c Th *(C, F, squares)* were cultured with M12c (Ia^d, *A, B, C*) or Balb/c cLC *(D, E, F)* that were unmodified *(open circles, open squares)* or TNP-modified *(solid circles, solid squares)*. After 3 *(C, F)* or 5 *(A, B, D, E)* d, proliferation was assessed by [^{3}H]thymidine incorporation. *Open triangles* in B designate stimulator cells alone; *solid triangles* in B designate stimulator cells plus allogeneic nonmodified Th.

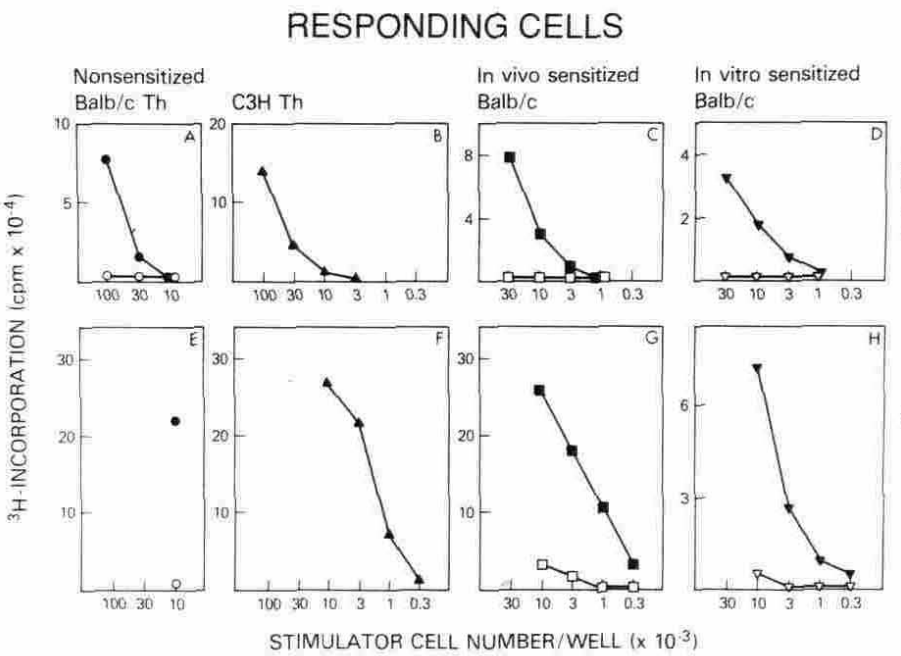

Figure 2. cLC and dendritic cell-enriched spleen cells are able to stimulate syngeneic nonsensitized Th when hapten modified. Nonsensitized Balb/c Th (10^5) depleted of autoreactivity *(A, E, round symbols)*, 10^5 freshly prepared C3 Th *(B, F)*, 10^5 Balb/c Th prepared from sensitized mice *(C, G)*, or 10^4 in vitro sensitized Th *(D, H)* were cultured with dendritic cell-enriched spleen cells *(A-D)* or cLC *(E-H)* that were unmodified *(open symbols in A, C, D, E, G, H)* or TNP modified *(solid symbols in A, C, D, E, G, H)*. After 3 *(C, D, G, H)* or 5 *(A, B, E, F)* d, proliferation was assessed by [^{3}H]thymidine incorporation.

geneic Th response in unprimed Th and could restimulate in vivo and in vitro generated Th cells that are hapten and MHC specific (Fig 2). Thus dendritic-cell enriched spleen cells, as well as cLC, in contrast to M12c, could stimulate vigorous primary Th proliferation.

Neither conditioned media derived from primary Th activation with TNP-modified cLC, conditioned media from spleen cells stimulated with concanavalin A, nor recombinant IL-2, were able to reconstitute Th proliferation to TNP-modified M12c cells (data not shown). Mixing TNP-modified M12c cells and TNP-modified cLC did not affect the proliferative response of previously nonactivated Th, thereby ruling out suppression by the hapten-modified M12c cells (data not shown).

Because density of class II MHC expression may relate to the antigen-presenting capacity of cells, flow cytometric studies were performed. Flow cytometric analysis of M12c cells and cLC revealed that M12c exhibited about 30% of the class II staining intensity of cLC (Fig 3). Dendritic cell-enriched spleen cells exhibited about 25% of the class II staining intensity of cLC (Fig 4). Thus, although the intensity of M12c and dendritic cell-enriched spleen cells for class II MHC expression are both lower than the intensity of cLC, there is a clear difference in their antigen-presenting capacity.

Characterization of a Th Subline that is Stimulated by Restricted APC Populations To determine whether there was any specificity with regard to the type of APC used for restimulation of the hapten-specific cell lines which resulted from primary in vitro sensitization with FITC-modified cLC, we tested a FITC-specific line after seven cycles and after 23 cycles of stimulation with FITC-modified cLC in 7-10-d intervals [2]. After seven cycles of stimulation, the mother line responded to all three types of FITC-modified APC (Fig 5*A*). When a subline of these FITC-specific T cells was tested after 23 cycles of stimulation, the line proliferated in response

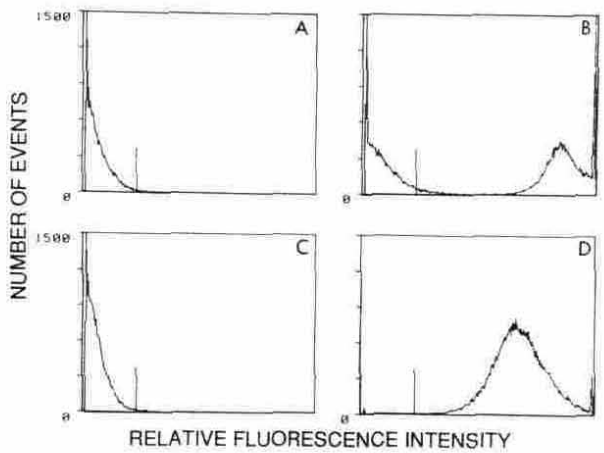

Figure 3. M12c cells show 30% of the MHC class II staining intensity of cLC. BALB/c cLC *(A,B)* or M12c cells *(C,D)* were incubated with FITC coupled 10.2.16 or MKD-6 antibodies. The analysis was performed on a FACS analyzer (Becton-Dickinson). Comparison of mean fluorescent channels of cLC and M12c cells indicate that the latter cell population exhibits a fluorescence intensity of 30% of the former.

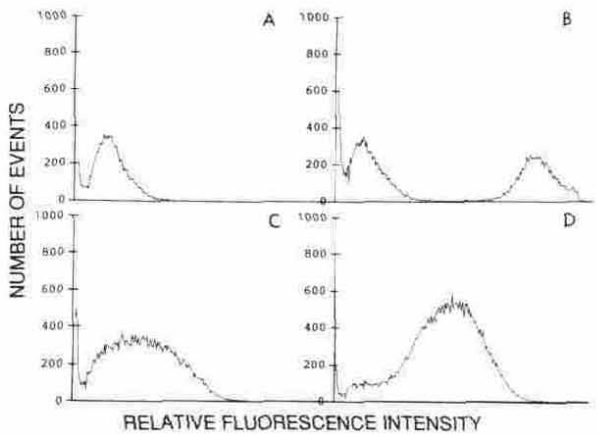

Figure 4. Dendritic cell-enriched spleen cells show 25% of the MHC class II staining intensity of cLC. BALB/c cLC *(A, B)* or BALB/c dendritic cell-enriched spleen cells *(C, D)* from Balb/c mice were first incubated with 10.2.16 (anti-Ia^k, *A, C*) or with MKD-6 (anti-Ia^d, *B, D*) monoclonal antibody and then with a F(ab)$_2$ goat anti-mouse IgG coupled to FITC. Analysis was done on an EPICS 5 (Coulter) flow cytometer. Comparisons of mean fluorescence channels of cLC and dendritic cell-enriched spleen cells indicate that the latter cell population had a fluorescent intensity 25% of that of cLC.

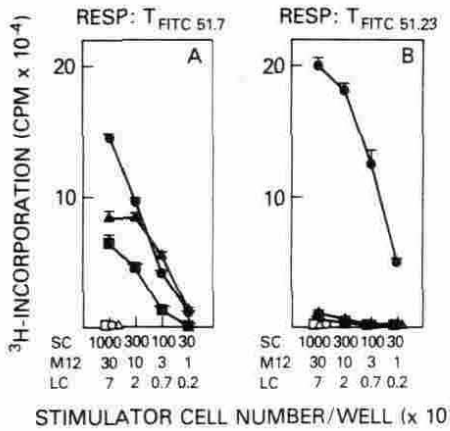

Figure 5. A long-term subline of a FITC-specific Th line responds to cLC but not M12c and spleen cells. Cells (10^4) from a FITC-specific Th line (after seven cycles of stimulation with FITC-modified cLC in 7-10d intervals) *(A)* and its subline (23 cycles of stimulation with FITC-modified cLC in 7-10d intervals) *(B)* were restimulated with FITC-modified spleen cells (SC, *solid triangles*), M12c (M12, *solid squares*), cLC *(solid circles)*, or unmodified cells *(open triangles, open squares, open circles)*. Proliferation was assessed by [^{3}H]thymidine incorporation after 3 d of culture.

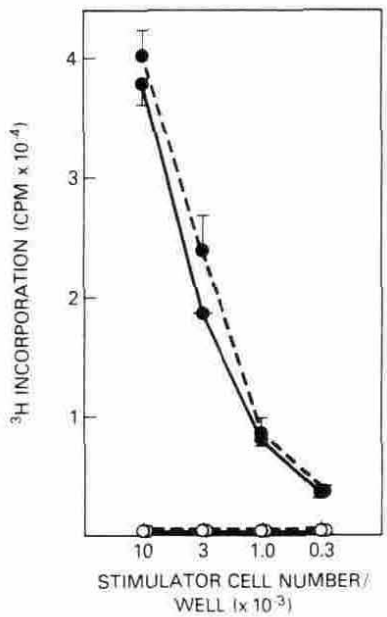

Figure 6. Depletion of Thy1$^+$ cells from cLC-enriched epidermal cells does not affect primary hapten-dependent Th proliferation induced by cLC. Cultured, nonadherent, viable epidermal cells were either treated with a monoclonal Thy1 antibody (IgM; New England Nuclear, Boston, MA) and complement *(solid lines)* or with complement alone *(dashed lines)* and were then either TNP-modified *(closed symbols)* or left unmodified *(open symbols)*. These cells were then used for stimulation of primary hapten-dependent T cell proliferation as described in *Materials and Methods*.

to FITC-modified cLC but not to FITC-modified spleen cells or M12c (Fig 5*B*). Upon restimulation, the subline responded to both FITC-modified cLC and FITC-modified dendritic cell-enriched spleen cells (data not shown). As seen in Fig 4, dendritic cell-enriched spleen cells exhibited only about 25% of the class II intensity of those of cLC. In comparison, M12c cells, to which the subline did not respond, showed about 30% of the class II intensity of cLC. Therefore, tissue derivation or differences in class II MHC intensity cannot totally explain the differential stimulation by various APC populations of the subline.

Thy1$^+$ Dendritic Epidermal T Cells Do Not Influence the Magnitude of Primary Th Proliferation Stimulated by cLC Because the cLC preparation before complement-mediated lysis contained 1%-2% Thy1$^+$ dendritic cells as assessed by indirect immunofluorescence microscopy, we determined whether depletion or addition of Thy1$^+$ dendritic epidermal T cells would influence the magnitude of primary Th proliferation. We found that cLC depleted of Thy1$^+$ cells with anti-Thy1 and complement stimulated Th to the same extent as cLC treated with complement alone when modified by TNP (Fig 6).

In the next two experiments, we added freshly prepared epidermal cells enriched for Thy1$^+$ dendritic epidermal T cells to either Th and syngeneic TNP-modified cLC (Table I, experiment 1) or to freshly prepared Th and allogeneic cLC (Table I, experiment 2). In both experiments, the addition of Thy1-enriched cells to the cultures did not enhance proliferation but rather inhibited to the same degree as did control cells consisting of Thy1$^-$ and MHC class II-depleted epidermal cells. The proliferative response of Thy1$^+$ dendritic epidermal T cells to concanavalin A, interleukin 1, and IL-2 suggests that these cells were functional (Table I). We therefore conclude that Thy1$^+$ dendritic epidermal cells do not significantly affect the magnitude of hapten-dependent or allogeneic Th proliferation induced by cLC.

Secondary Stimulation of Th with cLC Reveals Nonspecific Th Activation During Primary Th Activation Secondary stimulation of Th previously activated in vitro with hapten-modified cLC regularly resulted in hapten-specific responses when stimulated with spleen cells [1]. Th sensitized to TNP-modified cLC did not usually respond in secondary stimulation to FITC-modified cLC (Fig 7*A*). However, when cLC were used to restimulate Th primed in vitro to FITC-modified cLC cross-reactivity was observed in as much as these cells preferentially proliferated to FITC-modified cLC but also proliferated to a substantial degree to TNP-cLC (Fig 7*B*). We considered the possibility that TNP-specific Th were not activated in the first stimulation with FITC-modified cLC but only in the restimulation assay using TNP-cLC. If carry-over of nonactivated (thus high density) Th cells specific for TNP was responsible for the "nonspecific" response of the in vitro primed Th population, it would be expected that the low-density fraction of the primed Th would not respond to TNP-modified cLC upon restimulation. This possibility seemed unlikely, because low density (<65% Percoll) as well as high density (>65% Percoll) Th, which resulted from the culture of nonsensitized Th with FITC-modified cLC, showed the same degree of reactivity to the unrelated TNP-modified cLC upon restimulation (data not shown). Th lines generated by repeated stimulation with hapten-modified cLC usually responded in a hapten-specific manner [2].

Th Lines Generated In Vitro with Syngeneic Hapten-modified cLC Respond in a Hapten-specific Manner to Allogeneic cLC When established Th lines (>5 cycles of stimulation) were restimulated with syngeneic and allogeneic cLC, considerable proliferation of these lines was seen with allogeneic cLC that were modified with the relevant hapten (Fig 8*A*). Balb/c lines proliferated more vigorously in response to C3H cLC bearing the relevant hapten than C3H lines did in response to Balb/c cLC bearing the relevant hapten. Similar findings were observed using in vivo primed Th (Fig 8*B*). Overall, we tested five Th lines with allogeneic cLC and all showed hapten-specific proliferation. When we used a concentration of TNP 10 times lower for hapten modification, the magnitude of the response to syngeneic cells was the same, whereas it diminished in response to allogeneic cells (Fig 9). Hapten-specific Th clones with similar properties have been described [5,6].

DISCUSSION

Prior studies have indicated the importance of class II MHC molecules in the generation of primary in vitro Th proliferation using cLC [1]. The comparison of cLC and spleen cells enriched for dendritic cells in primary hapten-dependent Th proliferation (Fig 2) demonstrates that cLC are not unique in subserving this function. However, the comparison on a per cell basis is difficult to interpret because pure dendritic cell populations were not used. In addition,

Table I. Thy1$^+$ Dendritic Epidermal T Cells Do Not Affect the Magnitude of Primary Th Proliferation Induced by cLC

Stimulator/responder combination	Cells added	[^{3}H]thymidine incorporation[a] (mean cpm ± SEM)
Expt. 1 (hapten specific)		
10^4 C3H TNP-cLC + 10^5 C3H Th	None	69,066 ± 3,074
	2×10^4 C3H Thy1$^+$ (50%)	53,414 ± 3,074
	7×10^3	88,063 ± 7,812
	2×10^3	89,486 ± 3,202
	2×10^4 C3H Thy1$^-$ and Class II depleted	55,840 ± 1,504
	7×10^3	56,910 ± 252
	2×10^3	79,308 ± 2,230
10^4 C3H cLC + 10^5 C3H Th	None	300 ± 43
None	2×10^4 C3H Thy1$^+$	2,586 ± 43
None	2×10^4 C3H Thy1$^+$ (+ Con A + IL-1 + IL-2)[b]	18,192 ± 2,135
Expt. 2 (allogeneic)		
2×10^3 Balb/c cLC + 10^5 C3H T	None	161,383 ± 11,619
	3×10^4 C3H Thy1$^+$ (60%)	129,198 ± 29,515
	10^4	165,537 ± 11,610
	3×10^3	139,032 ± 8,871
	10^3	205,094 ± 2,023
	3×10^4 C3H Thy1$^-$ and class II-depleted	124,543 ± 17,120
	10^4	97,938 ± 12,782
	3 ± 10^3	116,069 ± 10,168
	10^3	166,050 ± 22,291
2×10^3 Balb/c cLC	None	456 ± 86
10^5 C3H T	None	405 ± 94
None	3×10^4 C3H Thy1$^+$	4,834 ± 1,299
2×10^3 Balb/c cLC	3×10^4 C3H Thy1$^+$	4,942 ± 139

[a] After 5 d of culture, proliferation was assessed by [^{3}H]thymidine incorporation.
[b] Con A: conconavalin A (2 μg/ml; Calbiochem, *San Diego, CA*); IL-1: recombinant human interleukin 1 beta (10 U/ml; Genzyme, Boston, MA); IL-2: 50 U/ml; Cetus, *Emeryville, CA*.

contaminating cells such as macrophages may suppress certain types of immune responses [7]. More surprising were the findings using the M12c cells as APC. Although able to stimulate primary allogeneic Th proliferation and to restimulate a hapten-specific Th line, primary Th proliferation with TNP-modified M12c was not observed in numerous attempts. It might be that our assay was not sensitive enough to observe Th activation stimulated by TNP-modified M12c. It is possible that the density of MHC class II molecules on M12c, which was lower than that of cLC (Fig 3), was too low to permit primary Th proliferation to TNP-modified M12c but was high enough to trigger the Th in the other two assays. However, arguing against this point are the experiments in which splenic dendritic cells, which also exhibit a lower density of class II molecules than cLC, were active in both assays of primary Th proliferation (Fig 4). The reason for the lack of hapten-dependent Th cell proliferation with M12c seems not to lie in their inability to stimulate nonsensitized Th proliferation, because allogeneic Th were clearly stimulated with M12c. Furthermore, M12c stimulated both high density (> 65% Percoll) and low density (< 65% Percoll) allogeneic Th (data not shown), excluding the possibility that only in vivo pre-activated Th were responding when stimulated with M12c. As well, M12c are able to present TNP, as shown by their restimulation of a TNP-specific Th line. The failure of M12c to stimulate primary hapten-dependent Th proliferation seems also not to reside in a deficiency in the production of soluble accessory signals, because addition of various recombinant cytokines or conditioned media did not reconstitute the response (data not shown). Another possible explanation for our findings with M12c is that the T-cell stimulatory determinant generated by TNP modification is so low on M12c that it can only stimulate Th with a high responder frequency, i.e., a hapten-specific line but not a nonsensitized Th population low in responder frequency. One more possible explanation for our results is that three separate activation mechanisms exist for the three assays used. cLC and dendritic cells would then possess the ability to subserve antigen-presenting function in all three, M12c in two of them (primary allogeneic Th proliferation and restimulation of sensitized Th) and macrophages in only one, i.e., restimulating Th (data not shown) . The experiments with the subline specific for FITC (Fig 5) confirm the notion that MHC class II density is not the simple decisive factor in terms of Th activation. Thus, dendritic cell-enriched spleen cells but not M12c were able to restimulate the subline, although both cell populations exhibit a similar class II intensity on flow cytometric analysis. Whether M12c and dendritic cell-enriched spleen cells provide a different set of secondary signals (soluble and/or contact mediated) for Th activation is not known.

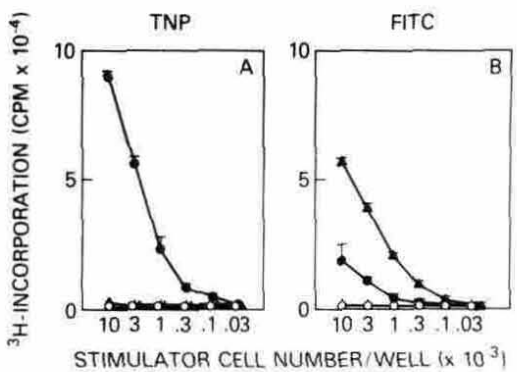

Figure 7. Restimulation of Th population previously activated by FITC-modified cLC reveals activation of T cells with specificity for TNP-modified cLC. Th (10^4) previously activated with cLC that were modified by TNP *(A)* or FITC *(B)* were restimulated with cLC that were unmodified *(open circles)* or modified with TNP *(solid circles)* or FITC *(solid triangles)*. Proliferation was assessed after 3 d of culture by [^{3}H]thymidine incorporation.

Although Thy1$^+$ dendritic epidermal T cells have been described as having a down-regulating function in vivo [8], we did not observe a significant inhibition in either primary Th proliferation assay. Thy1$^+$ dendritic epidermal T cells might therefore act by indirect mechanisms in vivo to modify Th responses.

Experiments utilizing secondary stimulation of Th previously activated in vitro with FITC-modified cLC revealed that Th proliferated to a lesser extent in response to cLC modified with the irrele-

vant hapten TNP (Fig 6*B*). This contrasts with restimulation experiments with spleen cells that showed hapten specificity [1]. The separation of high- and low-density Th clearly showed that activation of Th that respond upon restimulation with TNP-cLC occurred during primary stimulation, although hapten-dependent primary Th activation is clearly dependent on the presence of the specific Th within the non-primed Th population [1]. Taken together, these findings suggest that, during primary activation of Th with cLC, specific Th are activated along with a significant number of Th with irrelevant specificities. These Th are activated nonspecifically, i.e., without occupancy or triggering by the T-cell receptor. Similar phenomena can be detected after in vivo sensitization with regard to B- and T-cell responses [9–13] (Fig 8*B*). The possibilities that cross-reaction at the clonal level exists or that common new determinants on the APC are induced after hapten modification seems unlikely, because long-term Th lines always responded in a hapten-specific manner provided that carry-over of hapten-modified APC or their fragments is diminished by anti-class II and

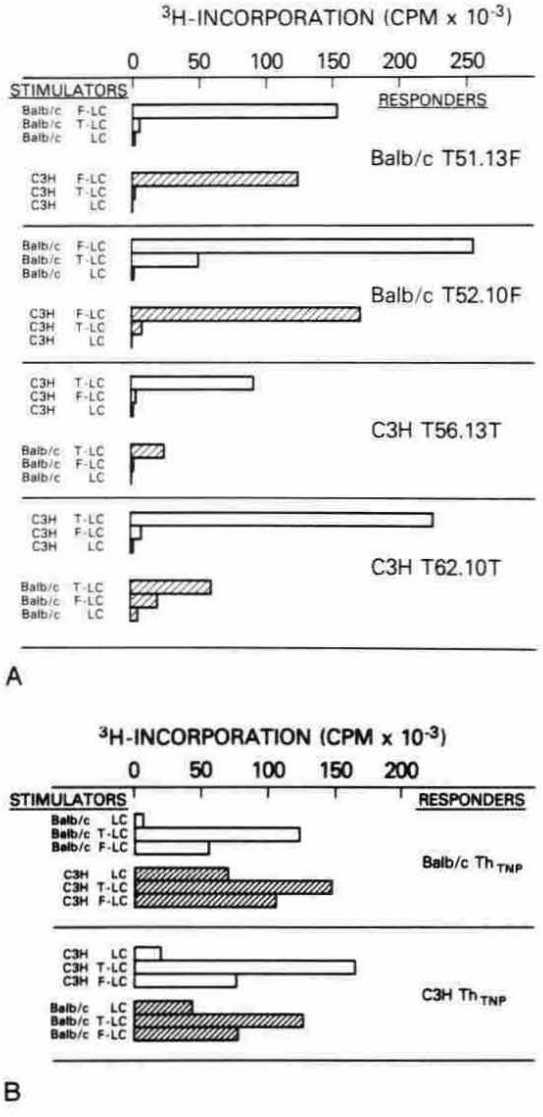

Figure 8. In vitro generated Th lines and in vivo primed Th do not show MHC restriction. *A*:cells (2 x 10^4) from Th lines with specificity for FITC (T51.13F, T52.10F) or TNP (T56.13T, T62.10T) were cultured with 10^4 syngeneic or allogeneic cLC that were either unmodified or modified with TNP (T-LC) or FITC (F-LC). *B*:Th (10^5) primed in vivo were cultured with 10^4 syngeneic and allogeneic cLC. cLC of Balb/c or C3H origin were used. Proliferation was assessed after 2 *(B)* or 3 *(A)* d of culture by [^{3}H]thymidine incorporation.

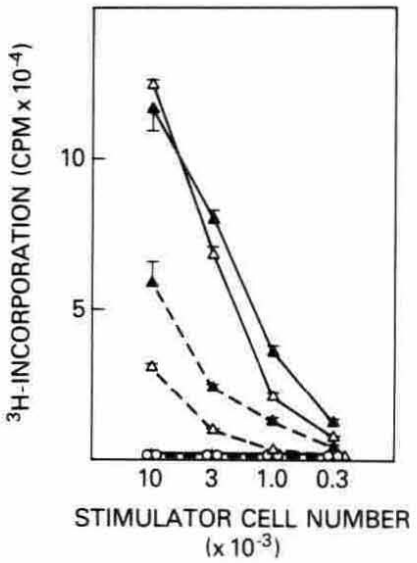

Figure 9. Allogeneic cLC show a greater dose dependence on hapten than syngeneic cLC for stimulation of hapten-specific Th lines. Syngeneic *(solid line)* or allogeneic *(dashed line)* cLC were unmodified *(open circles)* or modified with TNP at 0.1 *(open triangles)* or 1.0 *(closed triangles)* mM and used to restimulate 10^4 cells of a TNP-specific Th line generated in vitro. Proliferation was assessed after 3 d by [^{3}H]thymidine incorporation.

complement treatment and density gradient centrifugation of viable cells [2]. Nonspecific Th activation was only consistently observed when using FITC-modified cLC in the first and TNP-modified cLC in the second culture. This might be linked to the fact that primary Th proliferation with TNP-modified cLC was always stronger than using FITC-modified cLC. This suggests that the precursor frequency of TNP-specific Th is probably greater than that of FITC-specific Th. The detection of nonspecific Th activation is obviously facilitated when the previously activated Th population is restimulated with hapten-modified cLC that have a high Th precursor frequency.

The response of Th lines to allogeneic cLC modified with the relevant hapten was a constant finding and is also observed when using in vivo primed Th (Fig 8*A, B*). This occurrence has also been reported by other investigators using cloned Th [5,6]. These results might suggest that at least certain allogeneic class II molecules can serve as recognition elements for TNP-modified APC. This might indicate that the TNP carrier molecule can bind class II molecules of the Ia^k and Ia^d haplotype and can subsequently be recognized independently of the class II polymorphism. Alternatively, the class II molecule itself might be the "carrier" for haptens, as has been proposed by studies based on the cross-reactivity of TNP-specific clones with class II allogeneic APC [14]. In this case, we would postulate that non-polymorphic regions of the class II molecule serve as recognition elements. However, further studies are necessary to conclusively demonstrate whether the class II molecules are the immunologically relevant carriers in hapten-modified self-systems. Such studies would necessitate the use of a battery of clonal T cells. In addition, there is evidence for the existence of T cells that can recognize hapten alone [15]. It is therefore possible that our culture conditions favor the detection of such Th. However, the fact that the lines did not respond to hapten conjugated to soluble protein antigens (FITC-conjugated goat IgG, TNP-ovalbumin, data not shown) conflicts with the possibility that hapten-specific Th lines are able to recognize the hapten alone.

Taken together, these studies indicate that primary in vitro sensitization is dependent on factors beyond merely hapten modification of class II-bearing APC. The expanded cell lines which result are hapten-specific and, to a lesser extent, MHC specific. Mechanisms

which enable dendritic cell populations to become potent antigen-presenting cells for primary in vitro sensitization are currently under study.

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